POLYMERASE CHAIN REACTION-BASED DETECTION AND GENOTYPING OF MYCOBACTERIUM TUBERCULOSIS IN CLINICAL SPECIMENS

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

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IN

MICROBIOLOGY

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June 2025

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Subject : Regarding Practical Training/Internship for Six months of MSC Course.

Dear Madam,

With referene to your office letter No. DPMI/SIL/T&P/ASSC – 01 dated 20 Jan 2025, I would like to inform you that we were indeed happy to accept your request and confirm five months internship & training course on "MASTER IN SCINCE MICRO BIOLOGY (MSC. BIO)" in respect of Miss Simran Das, daughter of Mr. Sajal Kanti Das, commencing from 20 Jan 2025 at Anandaloke Sonoscan Centre Pvt Ltd, Siliguri.

Miss Simran Das has attended regular classes and training programme under the guidance of Dr. Sanjay Mallick, during the internship period she was involving various task including Laboratory Analysis, Data Collection, conduced at Anandaloke Sonoscan Centre and met all parameters of course. She is sober and keen to learn.

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled **"Polymerase Chain Reaction-Based Detection and Genotyping of *Mycobacterium tuberculosis* in Clinical Specimens"** in partial fulfilment of the requirements for the award of the degree of Master of Science in Microbiology is an authentic record of my own work carried out during the period of January-May 2025 under the supervision of Dr. Sanjay Mallick, department of Microbiology.

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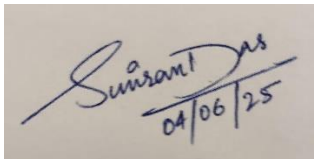

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TO WHOM IT MAY CONCERN

This is to certify that the project report titled "Polymerase Chain Reaction-Based Detection and Genotyping of Mycobacterium Tuberculosis in clinical specimens ", submitted by Ms. Simran Das (Registration No.230010316488, Enrolment No.2322211001031) to the Department of Microbiology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by her for the partial fulfilment of the requirements for the Master of Science degree.

Date: 06/06/2025

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Signature of Head of the Department

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ABBREVIATIONS

M. tuberculosis	*Mycobacterium Tuberculosis*
TB	Tuberculosis
PTB	Pulmonary TB
EPTB	Extra- pulmonary TB
LTBI	Latent Tuberculosis Infection (immune recognition of - and assumed infection with M. tuberculosis without clinical disease)
MDR-TB	Multi-drug resistant TB (i.e., drug resistant to at least isoniazid and rifampicin)
XDR-TB	MDR-TB, with additional resistance to: one of the injectable agents amikacin, kanamycin, or capreomycin - and to one of the fluoroquinolones
IGRA	Interferon gamma release assay - reflecting immune recognition of region of difference-1 coded proteins of M tuberculosis: ESAT-6 and CFP-10
TNF- α	Tumor necrosis factor alpha
TGF- β	Transforming Growth-Factor β
IFN- γ	Interferon-gamma
IL-10, IL-12	Interleukin 10, and 12
HIV	Human immunodeficiency virus
IRIS	Immune response inflammatory syndrome (denotes paradoxical response following cART in HIV-co-infected TB patients)
cART	Combined anti-retroviral therapy (HIV-treatment)
Vit D	Vitamin D
VDR	Vitamin D receptor
BCG	Live-attenuated M. bovis Bacille Calmette Guerin.
DOT	Directly Observed Therapy

ABSTRACT

Background:
Tuberculosis (TB) remains one of the world's leading infectious diseases, and the emergence of multidrug-resistant TB (MDR-TB), particularly resistance to rifampicin and isoniazid, has significantly complicated its management and control. Rifampicin resistance, which serves as a surrogate marker for MDR-TB, is primarily caused by mutations within the Rifampicin Resistance Determining Region (RRDR) of the *rpoB* gene. Rapid and accurate detection of rifampicin resistance is essential for timely initiation of second-line anti-TB treatment. This study aimed to assess the correlation between conventional phenotypic drug susceptibility testing (DST) and genotypic analysis targeting *rpoB* mutations in *Mycobacterium tuberculosis* (MTB) isolates.

Objectives:
To compare phenotypic and genotypic methods for the detection of rifampicin resistance in MTB isolates at initial, by detecting pcr based and genotyping in evaluating the mutation patterns within the *rpoB* RRDR region, and analyze their diagnostic and clinical relevance.

Methods:
A total of 37 MTB-positive clinical specimens were included in the study. Genotypic analysis was performed on four representative isolates through amplification and molecular characterization of the *rpoB* gene, focusing on codons 509–533, which constitute the RRDR. Mutation patterns were examined.

Results:
Out of the 37 MTB-positive cases, 10 (27%) were identified as rifampicin-resistant , 13 cases (35.1%) were found to be sensitive.. Genotypic analysis of the four selected isolates showed resistance-associated mutations within the RRDR of the *rpoB* gene. Case 01 exhibited a mutation at codon 521, Case 02 at codon 516, Case 03 at codon 526, while Case 04 showed a mixed mutation pattern involving multiple codons. All detected mutations were consistent with known resistance-associated regions, confirming the RRDR as a critical hotspot for rifampicin resistance. The presence of a mixed mutation pattern in one case suggested possible heteroresistance or mixed-strain infection, which could complicate phenotypic interpretation and treatment response.

Conclusion:
This study highlights a high degree of genotypic profiles in detecting rifampicin resistance in MTB. Genotypic methods offer rapid, sensitive detection of common mutations and are especially valuable in time-critical settings. Phenotypic methods, despite being slower, remain essential for confirming resistance expression and detecting non-RRDR-mediated resistance.

KEYWORDS

- *Mycobacterium tuberculosis*
- Rifampicin resistance
- *rpoB* gene mutations
- Rifampicin Resistance Determining Region (RRDR)
- Phenotypic drug susceptibility testing (DST)
- Genotypic resistance detection
- Molecular diagnostics
- Polymerase chain reaction (PCR)
- Drug-resistant TB
- Heteroresistance
- Mixed strain infection
- Rapid TB diagnosis
- Gene mutations
- TB control strategies
- Second-line anti-TB therapy
- Culture-based DST
- Diagnostic concordance
- Mutation hotspots
- Tuberculosis surveillance

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CHAPTER:01 INTRODUCTION

1.1 Overview of tuberculosis

Global and national burden

Tuberculosis (TB) is an infectious disease caused predominantly by the bacillus *Mycobacterium tuberculosis.* It typically affects the lungs (pulmonary TB) but can affect other sites as well (extra pulmonary TB) presents a significant global health challenge, evidenced by the staggering 10.8 million individuals who fell ill in 2023, marking the highest incidence recorded since global monitoring began in 1995. This resurgence has likely positioned TB as the leading cause of death from a single infectious agent globally in 2023, surpassing even COVID-19 after a three-year period, and it remains a critical factor in the mortality of people living with HIV and the growing crisis of antimicrobial resistance. Tragically, 1.25 million lives were lost to TB in 2023, including 161,000 individuals co-infected with HIV, underscoring the disease's devastating impact despite a slight decrease from the previous year. Geographically, the burden is disproportionately concentrated in the South-East Asian (46%), African (23%), and Western Pacific (18%) regions, with India (26%), Indonesia (10%), China (6.8%), the Philippines (6.8%), and Pakistan (6.3%) collectively accounting for over half of the world's TB cases in 2023. Notably, men constitute the majority of new cases (55%), followed by women (33%) and children and young adolescents (12%). The persistent threat of multidrug-resistant TB (MDR-TB) further complicates the landscape, with alarmingly low treatment access rates, as only around two in five affected individuals received necessary care in 2023.

Within India, the national burden of TB is the highest globally, although encouragingly, the country has witnessed a substantial decline in TB incidence, from 237 cases per 100,000 populations in 2015 to 195 per 100,000 in 2023, representing a 17.7% reduction. Similarly, TB-related mortality has decreased by 21.4% during the same period, falling from 28 to 22 deaths per lakh population. The National Tuberculosis Elimination Programme (NTEP) has achieved record-high case notifications, reporting 25.5 lakh cases in 2023 and 26.07 lakh in 2024, indicative of improved detection efforts. A significant achievement has been the reduction in missing TB cases from 15 lakhs in 2015 to a mere 2.5 lakh in 2023, an impressive 83% decrease. Driven by a strong commitment, India aims to eliminate TB by 2025, a bold target set five years ahead of the global 2030 goal. This ambitious endeavor is supported by a multi-pronged strategy under the NTEP, encompassing advanced diagnostic technologies, progressive policies, engagement of the private sector, financial assistance for patients through the Ni-kshay Poshan Yojana, proactive case finding initiatives, and robust multi-sectoral collaborations.

Pathogenesis of TB

The pathogenesis of TB unfolds through a sophisticated interaction between the *M. tuberculosis* bacterium and the host's immune system, commencing with the inhalation of infectious droplet nuclei expelled by individuals with active pulmonary TB. Upon reaching the alveoli, these bacilli encounter alveolar macrophages, the primary immune cells of the lungs. While macrophages typically engulf and destroy foreign pathogens, *M. tuberculosis* possesses remarkable survival mechanisms, including the ability to inhibit phagosome-lysosome fusion and neutralize the phagosomal environment, allowing intracellular survival and multiplication. This initial infection triggers the release of cytokines and chemokines, orchestrating the recruitment of other immune cells, such as monocytes, dendritic cells, and lymphocytes, to the site of infection. A hallmark of TB is the formation of granulomas, or tubercles, which are organized clusters of immune cells attempting to contain the infection and prevent bacterial dissemination. However, these granulomas also serve as a sanctuary for the bacteria, enabling them to persist in a latent state, often characterized by central caseous necrosis. The subsequent course of infection hinges on the host's immune competence. In most individuals with intact immunity, the

infection is contained, leading to latent TB infection (LTBI), where the person is asymptomatic and non-infectious but harbors dormant bacteria, often indicated by a positive TST or IGRA. Conversely, in individuals with compromised immunity, the granulomas may fail to contain the bacteria, leading to their multiplication and spread, resulting in active TB disease with characteristic symptoms like persistent cough, fever, night sweats, weight loss, and fatigue. In severe cases, particularly in vulnerable populations, the bacteria can disseminate hematogenously, causing miliary TB, a life-threatening condition marked by widespread dissemination of small tubercles throughout the body. A comprehensive understanding of this intricate pathogenesis is fundamental for the development of effective diagnostic tools, therapeutic interventions, and preventive strategies aimed at combating this enduring global health threat.

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1.2 Emergence of Drug Resistance TB

The escalating challenge of drug-resistant tuberculosis (DR-TB) represents a profound threat to global TB control efforts, undermining decades of progress in combating this ancient disease. The emergence and propagation of DR-TB strains are intricately linked to inadequacies in TB management practices, including suboptimal prescription patterns, erratic or interrupted treatment regimens, and, critically, the failure of patients to adhere to the full and prescribed duration of therapy. These programmatic weaknesses can be compounded by systemic issues such as inconsistent drug supply chains, limited accessibility to quality healthcare services, and socioeconomic factors that impede patient adherence. Furthermore, the transmissibility of these drug-resistant strains within communities perpetuates the epidemic, leading to increasingly complex, expensive, and often less successful treatment outcomes. The urgency in addressing DR-TB is underscored by its potential to reverse gains made in TB control and its contribution to increased morbidity and mortality among affected individuals.

1. c changes in the target protein, preventing or significantly reducing drug binding while preserving the protein's essential function for bacterial survival. Key examples include:
    - **Rifampicin Resistance (RIF-R):** Resistance to rifampicin is predominantly conferred by mutations within a specific 81-base pair "core region" of the *rpoB* gene, which encodes the beta subunit of RNA polymerase, the crucial enzyme inhibited by rifampicin. Different mutations within this region can result in varying levels of resistance. The high frequency of *rpoB* mutations makes molecular diagnostic tests for RIF-R highly reliable as a surrogate marker for MDR-TB.
    - **Isoniazid Resistance (INH-R):** Resistance to isoniazid, a prodrug activated by the bacterial catalase-peroxidase enzyme KatG, is more complex and can arise from mutations in several genes. The most frequently implicated gene is *katG*, where mutations can lead to a non-functional or less efficient enzyme, preventing the activation of isoniazid to its active form. Mutations in the *inhA* gene, encoding the enoyl-ACP reductase (a key enzyme in fatty acid synthesis and the target of activated isoniazid), and its promoter region (leading to overexpression of InhA, thus requiring higher concentrations of the drug) are also significant contributors to INH-R. Other genes, such as *ahpC* and *kasA*, can also be involved.
    - **Fluoroquinolone Resistance (FQ-R):** Resistance to fluoroquinolones, such as levofloxacin and moxifloxacin, primarily results from mutations in the *gyrA* and, less frequently, the *gyrB* genes. These genes encode the A and B subunits of DNA gyrase, a topoisomerase essential for DNA replication and repair. Mutations in specific "quinolone resistance-determining regions" (QRDRs) of these genes alter the gyrase structure, reducing its affinity for fluoroquinolones.
    - **Aminoglycoside Resistance:** Resistance to injectable second-line drugs like amikacin, kanamycin, and capreomycin can occur through various mechanisms, including mutations in the *rrs* gene, which encodes the 16S ribosomal RNA (a target of aminoglycosides), and mutations in genes encoding modifying enzymes such as aminoglycoside acetyltransferases (e.g., *eis* gene, particularly relevant for kanamycin resistance) and methyltransferases (e.g., *gidB*). These enzymes can chemically modify the aminoglycoside, reducing its ability to bind to the ribosome and inhibit protein synthesis.
2. Drug Activation Interference: (Disrupting the prodrug pathway) As mentioned earlier, some anti-TB drugs are administered as inactive prodrugs that require activation by specific bacterial enzymes to exert their antimicrobial effects. Mutations in the genes encoding these activating enzymes can lead to resistance by impairing or abolishing drug activation. The classic example is isoniazid, where mutations in the *katG* gene are a common mechanism of resistance.
3. Increased Efflux: (Pumping out the threat) *M. tuberculosis* possesses several efflux pump systems, which are transmembrane protein complexes that actively transport various molecules, including antimicrobial drugs, out of the bacterial cell. Upregulation of these efflux pumps or mutations that enhance their activity can lead to reduced intracellular drug concentrations, contributing to resistance against multiple drugs, including isoniazid, rifampicin, and fluoroquinolones. While efflux is often a contributing factor rather than the sole mechanism of high-level resistance, it can play a significant role in the development of low-level resistance and potentially facilitate the selection of mutations conferring higher levels of resistance.
4. Cell Wall Impermeability: (An intrinsic barrier) The unique and complex lipid-rich cell wall of *M. tuberculosis*, characterized by mycolic acids, arabinogalactan, and peptidoglycan, naturally presents a significant permeability barrier to many antimicrobial agents. While not typically the primary mechanism of acquired resistance to specific drugs, alterations in cell wall composition or integrity could theoretically contribute to reduced drug susceptibility. However, the specific genetic mechanisms underlying such changes in the context of acquired drug resistance are less well-defined compared to target modification and efflux.

1.3 Importance of early Diagnosis of MDR-TB

Impact on Patient outcomes

Early and accurate diagnosis of multidrug-resistant tuberculosis (MDR-TB) is paramount for both individual patient outcomes and broader public health considerations. The consequences of delayed or missed diagnosis can be devastating, leading to increased morbidity, mortality, and further transmission of drug-resistant strains.

- Improved treatment Success: Prompt diagnosis allows for the timely initiation of appropriate second-line drug regimens. These regimens, while longer and more toxic than those for drug-susceptible TB, offer the best chance of cure for MDR-TB patients. Delayed diagnosis often means delayed treatment, allowing the disease to progress and the bacteria to further damage the lungs and potentially spread to other organs, making successful treatment more challenging.
- Reduced Mortality: MDR-TB is associated with significantly higher mortality rates compared to drug-susceptible TB. Early diagnosis and treatment are critical for improving survival.
- Decreased Morbidity: Untreated or inadequately treated MDR-TB can lead to severe and irreversible lung damage, chronic illness, and a diminished quality of life. Early intervention can minimise these long-term consequences.
- Prevention of Further Drug Resistance Delayed or inappropriate treatment can lead to the development of additional drug resistance, potential resulting in extensively drug-resistant TB (XDR-TB), which is even more difficult and costly to treat, with significantly lower cure rates.
- Reduced Treatment Duration: While MDR-TB treatment is lengthy, early diagnosis can help ensure that patients are placed on the most effective regimen from the outset, potentially minimizing the overall duration of treatment compared to situations where ineffective first-line drugs are used for a prolonged period.
- Improved Patient Quality of Life: Early diagnosis and effective treatment can alleviate the debilitating symptoms of TB, such as cough, fever, weight loss, and fatigue, improving the patient's overall well-being and ability to carry out daily activities.

Public Health Implications

The public health implications of delayed MDR-TB diagnosis are far-reaching and threaten global TB control efforts.

Delayed diagnosis of MDR-TB has significant repercussions for public health. Each undiagnosed or mismanaged case serves as a potential source of further transmission within communities. This is particularly concerning given that MDR-TB is transmitted in the same way as drug-susceptible TB—through airborne droplets. The longer a person with MDR-TB remains undiagnosed and untreated, the greater the opportunity for them to infect others, potentially leading to a cascade of new MDR-TB cases. This not only increases the overall burden of drug-resistant TB but also strains healthcare systems, requiring more complex and costly treatment interventions. Furthermore, the spread of MDR-TB undermines the effectiveness of standard TB control strategies, making it more difficult to achieve global targets for TB elimination. Effective and rapid diagnostic tools, coupled with robust surveillance systems, are crucial for identifying MDR-TB cases early, interrupting transmission chains, and preventing the further amplification of this public health crisis.

- Increased Transmission: Undiagnosed and untreated individuals with MDR-TB continue to spread the resistant bacteria within their communities, leading to new infections.
- Higher Healthcare Costs: Treatment for MDR-TB is significantly more expensive than for drug-susceptible TB, placing a greater burden on healthcare systems.

- Strain on Healthcare Resources: Managing MDR-TB requires specialized facilities, trained personnel, and a consistent supply of second-line drugs, which can strain already limited healthcare resources, particularly in resource-limited settings.
- Undermining TB control effects: The spread of MDR-TB threatens to reverse the progress made in TB control over the past decades, making it more difficult to achieve global targets for TB elimination.
- Increased Morbidity and Mortality at the Population Level: The higher mortality rate associated with MDR-TB translates to a greater loss of life at the population level.

1.4 Need for Phenotypic and Genotypic Characterization

The accurate identification of drug resistance in *Mycobacterium tuberculosis* is critical for guiding appropriate treatment regimens and controlling the spread of DR-TB. Both phenotypic and genotypic drug susceptibility testing (DST) methods play crucial, albeit distinct, roles in this endeavor. Phenotypic DST, considered the traditional "gold standard" for most anti-TB drugs, directly assesses the ability of *M. tuberculosis* isolates to grow in the presence of specific concentrations of antimicrobial agents. Genotypic DST, on the other hand, detects specific genetic mutations in the *M. tuberculosis* genome that are known to be associated with drug resistance. While each method has its own set of advantages and limitations, their combined use often provides the most comprehensive and clinically relevant information for managing DR-TB.

Advantages and Limitations of Phenotypic Drug Susceptibility Testing

Advantages of Phenotypic DST-

- Phenotypic DST directly measures the organism's ability to survive and multiply in the presence of a drug, thus providing a functional assessment of resistance. This can capture resistance mechanisms that may not yet be fully understood at the genetic level or involve complex interactions of multiple genes.
- Theoretically, phenotypic DST can be performed for any anti-TB drug for which a critical concentration has been established, including newer and repurposed drugs where the genetic basis of resistance may not be fully elucidated.
- Phenotypic methods can sometimes detect heteroresistance, a situation where a bacterial population contains both drug-susceptible and drug-resistant subpopulations. This can have clinical implications for treatment outcomes.
- Phenotypic DST can be used to monitor a patient's response to treatment and detect the development of new drug resistances during therapy.
- For many anti-TB drugs, phenotypic DST remains the reference standard against which other DST methods are often compared.

Limitations of Phenotypic DST-

- *M. tuberculosis* is a slow-growing organism, and traditional phenotypic DST methods can take several weeks (typically 2-8 weeks) to yield results. This delay in diagnosis can lead to prolonged use of ineffective first-line drugs, disease progression, and continued transmission of resistant strains.
- Performing phenotypic DST requires well-equipped laboratories with biosafety facilities and highly trained personnel. This can limit its availability, particularly in resource-limited settings with a high burden of TB.
- The prolonged culture periods increase the risk of contamination, which can lead to invalid results.

- Phenotypic DST for some drugs, such as pyrazinamide (PZA) and ethambutol (EMB), can be technically challenging and may yield unreliable or poorly reproducible results.
- Phenotypic DST does not provide information on the specific genetic mutations responsible for resistance, which can be valuable for epidemiological studies and understanding resistance mechanisms.

Advantages and Limitations of Genotypic Drug Susceptibility Testing

Genotypic DST involves the detection of specific mutations in the *M. tuberculosis* genome that are known to confer resistance to particular anti-TB drugs. These tests can be performed on cultured isolates or directly on sputum samples in some cases.

Advantages of Genotypic DST-

- Genotypic DST methods, particularly molecular assays like Xpert MTB/RIF and line probe assays (LPAs), can provide results within hours or days, significantly faster than phenotypic DST. This allows for earlier initiation of appropriate treatment regimens.
- For well-characterized resistance mutations, genotypic DST can have high sensitivity and specificity, particularly for drugs like rifampicin and isoniazid.
- Identifying specific mutations can provide insights into the mechanisms of resistance and can be valuable for surveillance and epidemiological studies.
- Some genotypic tests can be performed directly on sputum samples, eliminating the need for prior culture and further reducing the time to diagnosis.
- While some advanced molecular techniques like whole-genome sequencing (WGS) require sophisticated infrastructure, simpler molecular assays can be implemented in moderately equipped laboratories.

Limitations of Genotypic DST-

- Current commercial genotypic DST assays typically target only the most common resistance-conferring mutations for a limited number of drugs. Resistance due to less common or novel mutations, or mechanisms not yet fully understood at the genetic level, may be missed.
- If resistance is conferred by a novel or uncharacterized mutation in a non-targeted gene region, genotypic DST will fail to detect it, potentially leading to false-susceptible results.
- The detection of a resistance-associated mutation does not always correlate perfectly with phenotypic resistance. Factors such as the specific mutation, its prevalence, and the presence of other modifying mutations can influence the level of resistance.
- While some advanced molecular methods like WGS can detect heteroresistance, simpler genotypic assays may not accurately quantify the proportion of resistant and susceptible subpopulations.
- The accuracy of genotypic DST relies on comprehensive and up-to-date databases linking specific mutations to drug resistance. These databases need continuous refinement as new resistance mechanisms are discovered.
- Techniques like WGS, while offering the most comprehensive genetic information, can be expensive and require significant bioinformatics expertise.

CHAPTER:02 REVIEW OF LITERATURE

2.1 History of Tuberculosis-Drug Resistance

The history of tuberculosis (TB) is a long and intertwined narrative of human suffering and scientific progress. Evident in skeletal remains from millennia past, TB has been a persistent companion to humanity, often referred to as "consumption" due to the wasting nature of the disease. Before the mid-20th century, the management of TB was largely palliative, relying on fresh air, rest in sanatoria, and nutritional support, which offered limited success against a disease that ravaged populations globally. The landscape of TB treatment underwent a monumental shift in 1944 with the ground-breaking discovery of streptomycin, the first antibiotic to demonstrate efficacy against *Mycobacterium tuberculosis*. This discovery by Albert Schatz, Selman Waksman, and their colleagues ushered in the modern era of TB therapy, offering hope where previously there was little. However, this initial triumph was soon tempered by the emergence of drug resistance, as bacteria within treated individuals began to survive streptomycin, leading to treatment failure and relapse. This early experience underscored a critical principle in combating TB: the propensity for resistance development when a single drug is used against the genetically diverse bacterial population.

The recognition of this challenge spurred the development and adoption of combination therapy, a strategy involving the simultaneous use of multiple anti-TB drugs to prevent the selection and proliferation of resistant mutants. The subsequent decades witnessed the introduction of several other pivotal first-line agents, including para-amino salicylic acid (PAS) in the late 1940s, often used alongside streptomycin; isoniazid (INH), discovered in the early 1950s, a highly potent bactericidal drug that became a cornerstone of both treatment and prophylaxis; and rifampicin (RIF), introduced in the 1960s, a powerful bactericidal agent that significantly shortened the overall duration of effective treatment. The addition of pyrazinamide (PZA) and ethambutol (EMB), also in the 1960s, further solidified the efficacy of combination regimens, paving the way for the widespread implementation of short-course chemotherapy, typically a six-month regimen involving isoniazid, rifampicin, pyrazinamide, and ethambutol. This standardized approach dramatically improved cure rates for drug-susceptible TB and became the global standard of care.

2.2 Mycobacterium Tuberculosis: Microbiological Characteristics

Structure

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis, possesses a unique set of microbiological characteristics that contribute to its pathogenicity, resilience, and the challenges associated with its diagnosis and treatment. It is a small, rod-shaped (bacillus) bacterium, typically measuring 0.5 to 3 micrometres in length and 0.2 to 0.5 micrometres in width. MTB is non-motile and non-spore-forming, and it is an obligate aerobe, meaning it requires oxygen for growth, which explains its predilection for the well-oxygenated upper lobes of the lungs. A defining feature of MTB is its slow growth rate, with a generation time of approximately 15-20 hours, leading to slow colony formation in culture (typically 2-8 weeks).

MTB is classified as an acid-fast bacillus due to its unique cell wall composition. Unlike Gram-positive or Gram-negative bacteria, the MTB cell wall is exceptionally rich in lipids, particularly mycolic acids (long-chain fatty acids), which account for up to 60% of its dry weight. This waxy, hydrophobic layer makes the bacterium resistant to Gram staining and many common antibacterial agents, as well as to harsh environmental conditions like drying and certain disinfectants. The acid-fastness is demonstrated using the Ziehl-Neelsen stain, where MTB retains the carbolfuchsin dye even after exposure to acid-

alcohol, appearing bright red under microscopy against a blue or green background. Other important components of the cell wall include arabinogalactan, a polysaccharide linked to mycolic acids, and peptidoglycan, providing structural integrity. The cell wall also contains various glycolipids and proteins that contribute to virulence and interact with the host immune system, such as cord factor (trehalose dimycolate), which is associated with virulence and the characteristic serpentine cording of MTB colonies in culture, and lipoarabinomannan (LAM), a major cell wall component that modulates host immune responses.

Genome Features

The genome of *Mycobacterium tuberculosis* is a single, circular chromosome typically around 4.4 million base pairs in length, encoding approximately 4,000 genes. It has a high guanine-cytosine (GC) content (around 65%), which contributes to the stability of its DNA and may influence its metabolism and adaptation.

Key feature includes-

- Extensive Lipid Metabolism Genes: A significant portion of the genome (around 9%) is dedicated to genes involved in lipid metabolism. This reflects the importance of lipid synthesis for the complex cell wall and the bacterium's ability to utilize lipids, including host-derived lipids like fatty acids and cholesterol, as carbon sources during infection, particularly in the nutrient-poor environment of the granuloma.
- PE/PPE Genes Families: MTB possesses two large families of genes, PE and PPE, which together account for about 10% of its coding capacity. These genes encode proteins with conserved N-terminal domains and variable C-terminal domains, and they are thought to play diverse roles in cell wall architecture, virulence, and modulation of the host immune response.
- ESX Secretion Systems: The genome encodes five Type VII secretion systems, known as ESX (ESAT-6 secretion system). These specialized protein export systems are crucial for the secretion of virulence factors, including the ESAT-6 and CFP-10 proteins, which play a role in macrophage escape, granuloma formation, and host cell damage.
- Absence of Plasmids and Limited Horizontal Gene Transfer: Unlike many other bacteria, MTB typically lacks plasmids and exhibits very limited horizontal gene transfer. This means that genetic diversity, including the development of drug resistance, primarily arises through spontaneous chromosomal mutations and their subsequent selection.
- Regions of Difference (RDs**):** Comparative genomics has identified specific genomic regions, known as Regions of Difference (RDs), that are present or absent in different strains within the *Mycobacterium tuberculosis* complex. These RDs can be used for strain typing, evolutionary studies, and understanding differences in virulence and host adaptation.
- Drug Resistance Genes: The genome contains genes that, when mutated, confer resistance to various anti-TB drugs. These include genes encoding drug targets (e.g., *rpoB* for rifampicin, *inhA* and *katG* for isoniazid, *gyrA* and *gyrB* for fluoroquinolones) or enzymes involved in drug activation or efflux. Understanding these genes and the specific mutations associated with resistance is crucial for genotypic drug susceptibility testing.
- Non-coding RNAs (ncRNAs): The MTB genome also encodes several non-coding RNA molecules that are involved in various regulatory processes, including gene expression and adaptation to different environmental conditions within the host.

2.3 Mechanism of Drug Resistance

Rifampicin resistance

Rifampicin (RIF) is a first-line anti-TB drug that targets DNA-dependent RNA polymerase, encoded by the rpoB gene. It binds to the β-subunit of RNA polymerase, inhibiting transcription.

The primary mechanism underlying this resistance is the acquisition of spontaneous mutations within the *rpoB* gene, which encodes the beta subunit of the essential RNA polymerase enzyme. This enzyme is the direct target of rifampicin, which normally functions by binding to it and effectively blocking RNA chain elongation, thereby inhibiting bacterial growth and leading to cell death. The vast majority, exceeding 95%, of clinically significant rifampicin resistance arises from alterations within a specific 81-base pair segment of the *rpoB* gene known as the rifampicin resistance-determining region (RRDR), located between codons 426 and 452 in *M. tuberculosis*. Mutations within this highly conserved region result in amino acid substitutions in the beta subunit of the RNA polymerase. These structural changes directly impact the rifampicin-binding pocket, reducing the affinity of the drug for its target. Consequently, even at therapeutic concentrations, rifampicin is unable to effectively bind and inhibit the altered RNA polymerase, rendering the bacterium resistant. Certain codons within the RRDR, particularly 516, 526, and 531 (using the widely referenced *E. coli* numbering), are hotspots for these resistance-conferring mutations. The presence of rifampicin resistance is a significant clinical indicator, as it is strongly associated with multidrug-resistant tuberculosis (MDR-TB), defined by concurrent resistance to at least rifampicin and isoniazid. While rare instances of rifampicin mono-resistance can occur, the detection of *rpoB* mutations typically necessitates treatment with more toxic and less effective second-line anti-TB drugs. Furthermore, while these *rpoB* mutations can sometimes impose a fitness cost on the bacterium, potentially affecting its growth or transmission, the evolution of compensatory mutations in other parts of *rpoB* or related RNA polymerase subunit genes can mitigate these fitness deficits, contributing to the persistence and spread of rifampicin-resistant strains. The strong correlation between *rpoB* mutations and phenotypic rifampicin resistance has made this gene a crucial target for rapid molecular diagnostic tests, such as the Xpert MTB/RIF assay, which can directly detect these mutations in clinical samples, enabling prompt diagnosis of RR-TB and facilitating the initiation of appropriate MDR-TB treatment regimens, ultimately improving patient outcomes and public health efforts to control this challenging form of the disease.

Isoniazid resistance

Isoniazid (INH) resistance is a complex phenomenon primarily driven by mutations in two key genetic loci: *katG* and the *inhA* operon. Isoniazid is a prodrug that requires activation by the bacterial catalase-peroxidase enzyme, encoded by the *katG* gene, to exert its ant mycobacterial activity. Once activated, isoniazid inhibits InhA (enoyl-ACP reductase), an enzyme crucial for the synthesis of mycolic acids, essential components of the mycobacterial cell wall.

Mutations in the *katG* gene are a major mechanism of high-level isoniazid resistance. The most frequently observed mutation is a Serine to Threonine substitution at codon 315 (S315T), which often leads to a reduction or loss of KatG enzyme activity. This impaired activation of isoniazid results in its ineffectiveness against the bacterium.

Resistance can also arise due to mutations affecting the *inhA* gene and its regulatory region. Mutations in the promoter region of the *inhA* gene, particularly the C-15T substitution, often lead to overexpression of the InhA enzyme. This increased production of the target enzyme can overwhelm the inhibitory effects of the activated isoniazid, resulting in low-level resistance. Mutations within the coding sequence of the *inhA* gene itself can also occur, altering the structure of the InhA enzyme and reducing its affinity for the isoniazid-NAD adduct, the active inhibitory compound.

The level of isoniazid resistance often correlates with the specific mutation. *katG* mutations typically confer higher levels of resistance compared to *inhA* promoter mutations. Interestingly, strains with *inhA* mutations may still be susceptible to higher doses of isoniazid, whereas those with *katG* mutations are generally resistant to all clinically relevant concentrations of the drug. Furthermore, *inhA* mutations can sometimes confer cross-resistance to ethionamide, another second-line anti-TB drug that also targets InhA. Understanding the specific *katG* and *inhA* mutations present in isoniazid-resistant isolates is crucial for guiding treatment decisions, as it can predict the likely level of resistance and potential for using higher doses of isoniazid or alternative drugs like ethionamide. Molecular diagnostic tests that target these genes are therefore vital for the rapid detection and management of isoniazid-resistant tuberculosis.

Other Minor Mutation

Beyond the well-established roles of *rpoB* in rifampicin resistance and *katG* and *inhA* in isoniazid resistance, a collection of other, less frequently encountered genetic mutations can also contribute to drug resistance in *Mycobacterium tuberculosis*. These "minor" mutations often result in lower levels of resistance compared to the primary drivers or may exert their effects through indirect mechanisms. While individually they might have a smaller impact, their presence can contribute to the overall drug resistance profile of a strain, potentially influencing treatment outcomes, especially in the context of multidrug resistance.

One notable gene in this category is ***ahpC***, which encodes alkyl hydroperoxide reductase, an enzyme involved in protecting the bacterium against oxidative stress. Mutations in the promoter region of *ahpC*, particularly insertions and deletions, are frequently observed in isoniazid-resistant strains. While *ahpC* is not a direct target of isoniazid, its overexpression due to these mutations is hypothesized to enhance the bacterium's ability to withstand the oxidative stress induced by isoniazid or its metabolites, thus contributing to resistance. Often, *ahpC* mutations occur in conjunction with *katG* mutations, possibly acting as compensatory mechanisms to alleviate some of the fitness costs associated with *katG* alterations.

Another gene of interest is *kasA*, encoding a β-ketoacyl-ACP synthase involved in the fatty acid synthesis pathway, which is closely linked to mycolic acid biosynthesis, the primary target of activated isoniazid. Mutations within the coding region of *kasA* have been associated with low-level isoniazid resistance. It's proposed that these mutations might subtly alter the mycolic acid synthesis pathway, reducing the effectiveness of isoniazid's inhibitory action.

Furthermore, mutations in *ndh*, encoding an NADH dehydrogenase involved in the electron transport chain, have also been linked to low-level isoniazid resistance. The precise mechanism is not fully understood but might involve alterations in the bacterium's metabolic state or the activation of isoniazid.

Beyond these, sporadic mutations in other genes involved in cell wall synthesis, such as those related to peptidoglycan production or the transport of cell wall components, have occasionally been implicated in contributing to isoniazid resistance. Similarly, mutations in regulatory genes or intergenic regions might indirectly affect drug susceptibility by altering the expression of various proteins involved in metabolism, stress response, or drug efflux.

Drug	Resistance Mechanism	Gene Involved	Notes
Rifampicin (RIF)	Mutation in RNA Polymerase Beta-subunit	rpoB	Mutation in the 81-bp (RRDR)
Isoniazid (INH)	Failure to activate the drug	katG	katG encodes catalase-peroxidase enzyme; S315T is the most common mutation
	Overexpression of target enzyme.	inhA promoter	inhA encodes enoyl-ACP reductase; C15T mutation leads to low-level INH resistance
Ethambutol (EMB)	Mutation affecting arabinosyl transferase	embB	Mutation at embB306 is most common
Pyrazinamide (PZA)	Impaired conversion to active form	pncA	pncA encodes pyrazinamidase; wide variety of mutations → variable resistance
Streptomycin (SM)	Mutation in ribosomal proteins or rRNA	Rpsl, rrs	rpsL: S12 protein (e.g., K43R); rrs: 16S rRNA
Fluoroquinolones	DNA gyrase mutations	gyrA, gyrB	gyrA: codons 90, 91, 94 often affected
Second-line injectable (amikacin, kanamycin, capreomycin)	Ribosomal Mutation or methylation	rrs, eis, tlyA	rrs A1401G mutation is common; eis promoter mutations also seen

Table 01 : Mechanism of Drug-Resistance in *Mycobacterium Tuberculosis*

Laboratory diagnosis of TB

The laboratory diagnosis of tuberculosis (TB) relies on a combination of methods to detect the presence of *Mycobacterium tuberculosis* and determine its drug susceptibility. Two fundamental techniques are smear microscopy and culture methods.

1. Smear Microscopy-
   This is a rapid and inexpensive initial diagnostic test. It involves examining a stained smear of a patient's sputum (or other clinical specimens) under a light microscope for the presence of acid-fast bacilli (AFB). *M. tuberculosis* is an acid-fast bacterium, meaning it retains the carbolfuchsin stain even after being treated with an acid alcohol solution.
2. Culture methods-
   Culture involves growing *M. tuberculosis* from a patient's specimen in a laboratory setting. This is considered the gold standard for the bacteriological confirmation of TB because it is more sensitive than smear microscopy and allows for species identification and drug susceptibility testing. Before culture, specimens (especially sputum) often undergo a digestion and decontamination process to liquefy the sample and kill contaminating bacteria and fungi that

grow faster than *M. tuberculosis*. Common agents used include N-Acetyl-L-cysteine (NALC) and sodium hydroxide (NaOH).
Culture isolates are essential for determining the susceptibility of *M. tuberculosis* to various anti-TB drugs. This is crucial for guiding treatment, especially in cases of drug-resistant TB. DST can be performed using both phenotypic (growth-based on solid or liquid media containing specific drug concentrations) and genotypic (molecular detection of resistance-conferring mutations) methods.

Feature	Smear Microscopy	Solid Culture	Liquid Culture (MGIT 960)
Time to result	Same day (few hours)	3-8 weeks	1-3 weeks
Sensitivity	Low (~50-60%)	Moderate	High
Detection threshold	$\geq 10^4$ bacilli/mL	10–100 bacilli/mL	10–100 bacilli/mL
Drug susceptibility	Not possible	Yes	Yes (automated)
Contamination risk	Low	Moderate	High if not properly decontaminated
Cost	Low	Low-moderate	high

Table 02: comparative overview of laboratory diagnosis

Both smear microscopy and culture methods play crucial roles in the laboratory diagnosis of TB. Smear microscopy is essential for rapid preliminary diagnosis and public health control measures, especially in high-burden areas. However, culture remains the definitive diagnostic tool due to its superior sensitivity and utility in drug resistance testing. A combination of both methods is recommended in most clinical settings to optimize diagnostic accuracy, speed, and treatment monitoring.

2.4 Phenotypic Drug susceptibility testing

Phenotypic Drug Susceptibility Testing (DST) is crucial for determining whether *Mycobacterium tuberculosis* isolates are susceptible or resistant to specific anti-tuberculosis drugs. This is typically done by observing the growth of the bacteria in the presence of different concentrations of these drugs. Two key methods and a fundamental concept in phenotypic DST are:

1. Portion method: This method, often considered a "gold standard" for phenotypic DST, compares the number of colonies growing on drug-containing media to the number of colonies growing on a drug-free control medium.
    - Procedure
        - A standardized inoculum of the *M. tuberculosis* isolate is prepared
        - This inoculum is inoculated onto a series of solid media (e.g., Lowenstein-Jensen or Middlebrook 7H10/7H11) containing specific concentrations of different anti-TB drugs and onto a drug-free control medium.
        - The media are incubated for several weeks (typically 3-4 weeks).
        - After incubation, the number of colonies on each drug-containing medium is counted and compared to the colony count on the control medium

- Interpretation: If the proportion of colonies growing on the drug-containing medium exceeds a predefined critical proportion (usually 1% of the growth on the control medium), the isolate is considered resistant to that drug at that specific concentration. If the proportion is below the critical value, the isolate is considered susceptible

- Advantages
  - Provides a quantitative measure of resistance within the bacterial population
  - Can detect mixed populations of susceptible and resistant bacilli.
- Disadvantages
  - Labor-intensive, requiring manual colony counting.
  - Slow turnaround time due to the slow growth of *M. tuberculosis* on solid media.

2. Critical concentration
   The critical concentration is the lowest concentration of an anti-tuberculosis drug that inhibits the growth of a high percentage (typically 95-99%) of wild-type (drug-susceptible) M. tuberculosis strains while allowing the growth of most drug-resistant strains.
   In phenotypic DST, particularly when using a single concentration of a drug (as is often done in automated systems like MGIT 960 or even in simplified manual methods), the concept of critical concentration is essential.

Drug	LJ Medium (µg/mL)	MGIT 960 (µg/mL)
Isoniazid (INH)	0.2/1.0	0.1/0.4
Rifampicin (RIF)	40	1.0
Ethambutol (EMB)	2.0	5.0
Streptomycin	4.0	1.0
Ofloxacin	-	2.0
Amikacin	-	1.0

Table 03: Example of Critical concentration (as per WHO and CSLI guidelines)

Note: For Isoniazid (INH), two concentrations are often used to distinguish low level and high level resistance.

2.5 Molecular Methods for Detecting Drug-Resistance

The advent of molecular methods has ushered in a transformative era in the diagnosis and management of drug-resistant tuberculosis (DR-TB). These techniques, which directly interrogate the *M. tuberculosis* genome for resistance-conferring mutations, offer significant advantages in terms of speed and potential scope compared to traditional phenotypic drug susceptibility testing (DST). This rapid identification of resistance allows for the timely initiation of effective second-line treatment regimens, ultimately improving patient outcomes and curbing the transmission of DR-TB strains.

<u>GeneXpert MTB/RIF and its Advancements:</u>

The GeneXpert MTB/RIF assay integrates sample processing, nucleic acid amplification, and target detection in a self-contained cartridge. Sputum or other respiratory samples are mixed with a reagent that lyses bacterial cells and releases DNA. This lysate is then transferred to the cartridge, which contains primers for amplifying a specific region of the rpoB gene (the RRDR) and fluorescently

labelled probes that hybridize to both wild-type and common mutant sequences within this region. Real-time PCR is performed within the cartridge, and the fluorescence signals are monitored by the GeneXpert instrument. The presence of M. tuberculosis DNA is indicated by the amplification of a specific target sequence (often IS6110 or other conserved regions). Rifampicin resistance is inferred from the failure of the probes specific to the wild-type RRDR sequence to hybridize, coupled with the potential hybridization to mutant-specific probes.

Strengths for Research: Its ease of use, rapid turnaround time (within 2 hours), and relatively high sensitivity and specificity for detecting both M. tuberculosis and rifampicin resistance make it invaluable for epidemiological studies, rapid screening in high-burden settings, and point-of-care diagnostics. Its standardized platform ensures reproducibility across different laboratories.

Limitations for Research: While highly effective for rifampicin resistance, the original assay only targets a limited region of rpoB and may miss rare mutations outside this hotspot. Furthermore, it does not provide information on resistance to other crucial drugs like isoniazid or second-line agents.

Advancements: Newer versions, such as Xpert MTB/XDR, are being developed to simultaneously detect resistance to isoniazid and fluoroquinolones, addressing some of the limitations of the original assay. Research is ongoing to expand the panel of detectable resistance mutations for a wider range of drugs on similar platforms.

Real-time PCR: Beyond Simple Detection

Real-time PCR allows for the quantification of DNA amplification as it occurs. For drug resistance detection, various strategies can be employed. Allele-specific PCR uses primers that are perfectly complementary to either the wild-type or a specific mutant sequence. Amplification occurs preferentially when the primer matches the target. Melting curve analysis can differentiate between wild-type and mutant sequences based on their different melting temperatures. Fluorescently labelled probes, such as TaqMan probes, can be designed to hybridize specifically to wild-type or mutant sequences, releasing a fluorescent signal upon amplification only when the target is present.

Strengths for Research: Real-time PCR offers high sensitivity and specificity and can be adapted to detect virtually any known mutation. Its quantitative nature can potentially be used to assess mixed populations of resistant and susceptible bacteria. It is also amenable to automation and high-throughput analysis.

Limitations for Research: Designing and validating specific assays for each mutation of interest can be time-consuming and resource-intensive. It often requires specialized equipment and expertise in assay development and data analysis.

Advancements: Ongoing research focuses on developing multiplex real-time PCR assays that can simultaneously detect multiple resistance mutations, improving the speed and efficiency of the technique for broader application.

2.6 Previous Studies on MDR-TB Characterization

Numerous studies conducted globally and regionally have contributed significantly to the understanding of Multidrug-Resistant Tuberculosis (MDR-TB), particularly in terms of its epidemiology, genetic mutations, resistance mechanisms, and treatment outcomes. Globally, large-scale surveillance data from the World Health Organization (WHO) and research consortia like the Global Project on Anti-Tuberculosis Drug Resistance Surveillance have shown that MDR-TB accounts for approximately 3–4% of new TB cases and 18–20% of previously treated cases, with the highest burden reported in

countries like India, China, Russia, and South Africa. Molecular characterization of MDR-TB strains from countries such as Peru, South Korea, and Eastern Europe has revealed high frequencies of rpoB mutations (especially at codon 531) conferring rifampicin resistance, and katG (S315T) and inhA promoter mutations associated with isoniazid resistance. Studies have also highlighted the emergence of pre-XDR and XDR-TB, particularly in Eastern Europe and Central Asia, with resistance extending to fluoroquinolones and injectable second-line drugs. These findings underscore the urgent need for rapid molecular diagnostics, drug susceptibility testing, and individualized treatment regimens.

CHAPTER:03 AIMS AND OBJECTS

3.1 Aims

This study aimed to evaluate the effectiveness of RT-PCR-based genotypic methods, particularly targeting the rpoB gene, in detecting rifampicin resistance among clinical isolates of Mycobacterium tuberculosis." The study seeks to evaluate the drug resistance patterns of clinical isolates against first-line anti-tubercular drugs—specifically isoniazid (INH) and rifampicin (RIF).

Molecular (genotypic) methods will be employed to identify the specific genetic mutations conferring resistance, primarily within the rpoB gene (for rifampicin)

3.2 Specific Objectives

1. To isolate *Mycobacterium Tuberculosis* (MTB) from clinical specimens collected from hospitalized patients suspected of pulmonary or extra pulmonary tuberculosis.
2. To detect gene mutations associated with drug resistance in phenotypically resistant isolates through genotypic methods

CHAPTER:04 MATERIALS AND METHODS

4.1 Study design and duration

This study was designed as a cross-sectional, observational, and laboratory-based investigation aimed at characterizing multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) isolates from clinical samples of hospitalized patients. The study was carried out in the Department of Microbiology at Anandaloke Sonoscan Centre Siliguri, a multispecialty and referral hospital located in Siliguri, West Bengal, India. The duration of the study spanned a period of three-four months, from January 2025-April 2025.

The cross-sectional design was chosen because it is suitable for assessing the burden and characteristics of drug resistance at a specific point in time without following patients longitudinally. The observational nature of the study means that there was no intervention in patient treatment; rather, clinical samples received as part of routine diagnostic workup were used for laboratory investigations, ensuring minimal disruption to clinical care. This design allowed for an efficient assessment of the prevalence of phenotypic resistance, the distribution of key resistance-conferring mutations, and the concordance between phenotypic and genotypic methods within a defined hospital population.

4.3 Sample collections and processing

Types of clinical samples collected: Clinical specimens were obtained from hospitalized patients with suspected or confirmed tuberculosis who were admitted to various departments within the tertiary care hospital. A wide range of pulmonary and extra pulmonary samples were included in the study to ensure a comprehensive representation of TB manifestations.

- Pulmonary specimens:
    - Sputum (early morning, expectorated or induced)- Early morning sputum (deep cough originating from the lungs, not saliva) samples were collected in a sterile, wide-mouthed, leak-proof container after instructing the patient to rinse the mouth with water to reduce contamination.
    - Broncho alveolar lavage (BAL)- Obtained by a pulmonologist via bronchoscopy under aseptic conditions and collected into a sterile container.
    - Tracheal aspirates- Collected aseptically via suction through an endotracheal or tracheostomy tube into sterile mucus traps.
- Extra-pulmonary specimens:
    - Pleural Fluid- Aspirated by thoracentesis under sterile conditions into sterile containers.
    - Cerebrospinal fluid (CSF)- Collected by lumbar puncture under aseptic precautions.
    - Ascitic fluid- Obtained via paracentesis under sterile technique and centrifuged to concentrate bacilli
    - Lymph node aspirates- Collected by trained clinicians under aseptic conditions using fine-gauge needles. Direct smears prepared from aspirates. Remaining material suspended in phosphate buffer or saline and processed using NALC-NaOH, if required.
    - PUS- Collected from lymph nodes or abscesses using sterile syringes or swabs. If thick, pus was diluted with sterile saline and subjected to NALC-NaOH decontamination.
    - Synovial and pericardial fluids- Aspirated under aseptic conditions by clinicians.

Each sample was collected in sterile, leak-proof, wide-mouth containers using aseptic precautions. All specimens were transported to the Microbiology Laboratory as soon as possible, preferably within 2

hours of collection, and were processed promptly to preserve the integrity of potential *Mycobacterium tuberculosis* (MTB) bacilli.

Decontamination

Spilling of potentially infectious material were cleaned up immediately with absorbent paper tissue and the contaminated area were decontaminated with disinfectants such as 0.5% freshly prepared sodium Hypochlorite [10 times dilution of 5% Sodium hypochlorite (household bleach) before continuing the work. Sodium hypochlorite was not used on an acid-containing spill unless the spill-area was wiped dry first. Materials used to clean spills, including gloves were disposed off potentially bio-hazardous waste e.g. in a biohazard waste container.

4.4 Principle of Truenat® System: DNA Extraction and Microchip-Based RT-PCR Detection

The Truenat® system is a portable, battery-operated, real-time PCR platform designed for rapid, accurate, and decentralized diagnosis of tuberculosis (TB) and multidrug-resistant TB (MDR-TB). It comprises two main components:

A. Truenat® Prep Kit – for sample lysis and DNA extraction

B. Truenat® Micro PCR Chip – for amplification and detection of TB DNA and associated resistance genes (e.g., *rpoB* for rifampicin resistance)

DNA Extraction using Trueprep® Auto Universal Cartridge Based Sample Prep Kit

Trueprep® Auto Universal Cartridge Based Sample Prep Kit protocol uses a proprietary matrix enclosed in a cartridge to purify nucleic acids from clinical samples. The cartridge also contains pre-loaded Internal Positive Control (IPC). The IPC is a full process control that undergoes all the processes the specimens undergo, from extraction to amplification, thereby validating the analysis from sample to result.

Materials provided-

A. The Reagent pack contains the following:
   a. Reagents

No	Contents	Purpose
1.	Wash Buffer A	To wash inhibitors from the sample- Green CAP
2.	Wash Buffer B	To wash inhibitors from the sample- Blue CAP
3.	Elution Buffer	To elute nucleic acids- White CAP
4.	Priming Waste	To purge residual liquid from tubing- Red CAP

   b. Reagent Reset Card-1 No.

B. The cartridge Pack contains the following

No.	Contents	Purpose
1.	Cartridge	Cartridge containing immobilized internal control (IPC) for extraction
2.	Elute collection tube (ECT)	Capped tubes for collection and storage of extracted nucleic acids
3.	Elute collection tube (ECT) label	To label elute collection tube (ECT)
4.	Disposable transfer pipette	To pierce the seal of elute chamber and to transfer extracted nucleic acids from the elute chamber of cartridge into the elute collection tube (ECT)

Table 04: Reagents and cartridge pack

C. Disposable transfer pipettes (graduated)- 3ml
D. Package insert

Extraction procedure-

1. Lysis of the bacterial cells: The sample is treated with a proprietary Lysis Buffer that contains chaotropic agents and detergents that, disrupt the lipid-rich mycobacterial cell wall (rich in mycolic acids) of *M. tuberculosis*, which is otherwise resistant to conventional lysis. It also denatures proteins and inactivate nucleases that could degrade DNA. The mixture is incubated at room temperature to ensure complete lysis.
2. Binding of DNA to Magnetic Beads: Magnetic silica coated beads are added to the lysed sample along with a binding buffer. Under high salt-concentration and optimal pH, negatively charged DNA molecules bind to the positively charged silica surface of the beads via electrostatic interaction. The suspension is incubated briefly to ensure maximum binding to DNA to beads.
3. A cartridge is taken from the Trueprep auto universal cartridge based sample prep kit, labelled as the patient ID, date, in the space provided on the cartridge label and placed the cartridge in the stand provided with the device. After the sample been pre-treated as mentioned above as per sample type and transfer the entire content using disposable transfer pipette (graduated-3ml) into the sample chamber of the cartridge and placed the cartridge into the device, gently closing the door. The device performs all the steps automatically.
    - Washing: Wash Buffer A is added to remove the cellular debris, protein contaminants.
    - After magnetic separation the supernatant is discarded.
    - Wash Buffer B (usually ethanol based) is added for further purification
    - This step is repeated for 1-2 times to ensure complete removal of PCR inhibitors.
    - Elution of DNA: after final washing and magnetic separation, an elution buffer is added. This buffer (typically low salt tris-EDTA or nuclease free-water) releases from DNA from the silica beads.
4. Meanwhile, mentioning the patient ID, date in the space provided on the elute collection tube (ECT) label provided in the cartridge pouch and affix the label to the ECT. As the process is conducted by the beep sound from the device and automatic ejection of cartridge. Taking out the cartridge and placing on the stand. Then by piercing the seal of the elute chamber and aspirate of the entire elute into ECT using disposable transfer pipette.

Real-Time Amplification and Detection of MTB-DNA and Drug Resistance Mutations

The target sequence for this kit are nrdz gene (that codes for the ribonucleoside-diphosphate reductase adenosyly cobalamin-dependent protein) and IS6110 gene sequence. The regions selected are specific to the MTB complex.

Contents of the Truelab® MTB Plus Kit-

A. Individually sealed pouches
B. Package insert: Truelab® MTB Plus micro PCR chip, Microtube with freeze dried PCR reagents, DNase and RNase free pipette tip and desiccant pouch.

The micro PCR chip is stable for two years from the date of manufacture if stored between 2-30 degree centigrade. It is stable for up to one month at temperatures up to 45 degrees centigrade. It is not supposed to freeze or exposure to light or elevated temperatures.

1. Loading the Micro PCR Chip: The Truenat® MTB Plus chip is placed on the chip tray of the Truelab® Real Time Quantitative micro PCR Analyser. The chip contains lyophilized PCR reagent including- specific primers for *M. tuberculosis* (IS61100), Taq DNA polymerase, sequence specific hydrolysis probes with fluorescent reporters and internal amplification controls (IAC) to monitor reaction validity.

Figure 01: Micro-chip freeze and DNA extracted in an eluted

Figure 02: Truelab MTB Plus micro-PCR chip with sample loading

2. Sealing and Insertion into the Truelab Analyser: The chip is sealed with a transparent adhesive cover to prevent contamination and evaporation. Six (6) µL of the purified DNA is then dispensed using the provided micropipette and tip into the microtube containing freeze dried PCR reagents and allowed to stand for 30-60 seconds to get the clear solution. *(No mixing by tapping, shaking or by reverse pipetting was done)* Six (6) µL of the clear solution is then pipette out using the same pipette and tip and dispensed into the reaction well of the Truenat® Micro PCR Chip and the test is started.

3. Real Time PCR Amplification: The analyser carries out thermal cycling: Denaturation (~95°C): DNA strands separate. Annealing (~55–60°C): Primers and probes bind to their complementary sequences. Extension (~72°C): DNA polymerase synthesizes new DNA strands.
   During extension: The Taq polymerase cleaves the probes hybridised to the target sequence. This release the fluorescent dye from the quencher, producing a detectable fluorescent signal. This signal increases proportionally with DNA amplification.

4. Real-Time Detection: The fluorescent is detected at each cycle. The system plots a Ct (cycle threshold) value- the point where the fluorescence crosses a present threshold. A low Ct values indicates a higher bacterial load; a high Ct value suggest a lower DNA concentration.
   For drug resistance detection: Probe for wild type and mutant alleles are used. A drop in a wild type signal or a positive signal for mutant probe indicates rifampicin resistance.

CHAPTER:05 RESULTS

5.1 Demographic Profile of patients

The demographic characteristics of patients enrolled in the study were recorded and analysed. The key parameters included age, gender, and clinical diagnosis based on symptoms, radiographic findings, and clinical suspicion of tuberculosis.

Patients ranged from **0 to >60** years of age, with the majority falling within the **15-30** age group.

A total of 26 males **(43.3%)** and 34 females **(56.7%)** were included in the study. A slight female predominance was observed.

Patients were suspected of pulmonary or extra-pulmonary tuberculosis based on presenting symptoms such as:

- Persistent cough for more than 2 weeks
- Hemoptysis
- Fever, night sweats
- Weight loss
- Radiographic findings suggestive of TB
- Clinical suspicion in immunocompromised patients (e.g., HIV)

Demographic factors	**MDR-TB (n= 60)**	**Percentage (%)**
Sex		
Female	**34**	**56.7%**
Male	**26**	**43.3%**
Age-years		
0-14	**2**	**3.3%**
15-30	**22**	**36.7%**
31-45	**4**	**6.7%**
46-60	**15**	**25.0%**
>60	**17**	**28.3%**

Table 05: Age Group Distribution of MDR-TB Patients

5.2 Sample-wise Distribution

This distribution reflects the variety of pulmonary and extra-pulmonary specimens routinely tested in the clinical microbiology laboratory for detection of *Mycobacterium tuberculosis* and drug resistance**.**

Sample type	Total Number of samples	Percentage %
Sputum	**3**	**5%**
Pleural fluid	**12**	**20%**
Lymph node aspirate	**18**	**30%**
Cerebrospinal fluid	**6**	**10%**
Bronchoalveolar lavage (BAL)	**8**	**13.3%**
PUS	**4**	**6.7%**
Tracheal aspirate	**4**	**6.7%**
Synovial or pericardial fluid	**5**	**8.3%**

Table 06- Sample-wise distribution of the clinical sample

5.3 Drug Resistance Profile

The target sequence of this kit is the RRDR region of the rpoB gene and the codon region coverage is MTB codon Numbering [H37Rv]: codon 428-435 & codon 455-445-452 (E.coli codon numbering: codon 509-516 & codon 526-533), representing mutation hotspots known to be related to rifampicin resistance.

A total number of 37 positive MTB cases were recorded from the screened patient cohort during the study period. The Rifampicin resistance profile was assessed and the findings are summarised below.

Rifampicin susceptibility	Number of cases positive (n=37)	Percentage %
Sensitive	13	35.1%
Resistance (presumptive MDR-TB)	10	27.0%
Indeterminate	8	21.6%
Not detected/ unavailable	6	16.2%

Table 07: Drug resistance profile

5.4 Genotypic Mutation Interpretation (based on Ct shift Patterns)

P1 and P2 likely refers to amplification curves of different probes in the rpoB gene region (rifampicin-determining region or RRDR) Control is the internal positive control to validate that the test worked correctly.

Case 01-

Lymph node aspirate	Rifampicin Detected	P1= 66.49	P2= 66.21	Control= 71.54	rpoB codon 531

Case 02-

Cerebrospinal fluid	Rifampicin detected	P1=66.77	P2= 64.52	Control= 70.66	rpoB codon 516

Case 03-

Lymph node aspirate	Rifampicin Detected	P1= 66.78	P2= 68.82	Control= 71.58	RpoB codon 526

Case 04-

Lymph node aspirate	Rifampicin detected	P1=66.88	P2=64.76	Control= 71.98	Mixed pattern

CHAPTER 06: DISCUSSIONS

6.1 Interpretation of key findings.

The genotypic analysis performed in this study focused on detecting mutations within the rifampicin resistance-determining region (RRDR) of the rpoB gene in four representative Mycobacterium tuberculosis isolates. All four cases exhibited mutations within the RRDR (codons 509–533), confirming the presence of rifampicin resistance-associated genetic alterations.

Specifically, mutations were observed at codon 521 (Case 01), codon 516 (Case 02), and codon 526 (Case 03), while Case 04 demonstrated a mixed mutation pattern, indicating either heteroresistance or co-infection with multiple strains. These findings are consistent with globally reported mutation hotspots associated with rifampicin resistance. Mutations at codons 516 and 526 are among the most frequently encountered, while codon 521, though less commonly reported, is still within the RRDR and contributes to resistance.

Genotypic methods, particularly those targeting the RRDR of the rpoB gene, allow rapid identification of rifampicin resistance by detecting specific mutations associated with resistance. These assays, including PCR-based platforms and line probe assays, have become integral to TB control programs due to their speed and accuracy.

Strength:

- Rapid turnaround time, often delivering results within 24–48 hours.
- High specificity and sensitivity for the most common resistance-conferring mutations (especially in codons 516, 526, and 531).
- Can detect mutations even before phenotypic resistance becomes evident.

Limitations:

- May not detect rare or novel mutations outside the targeted regions, leading to false-negative results.
- Cannot differentiate between silent mutations and those that confer resistance unless sequencing is performed.
- May miss cases of phenotypic resistance due to non-mutational mechanisms.
- Interpretation can be complicated by the presence of mixed strains or heteroresistance.

CHAPTER 07: CONCLUSION

A total of 37 MTB-positive cases were analysed, of which 27% were identified as rifampicin-resistant by phenotypic DST. Genotypic analysis was conducted on four representative isolates, all of which revealed mutations within the rifampicin resistance-determining region (RRDR) of the *rpoB* gene.

This study successfully demonstrates the application of real-time polymerase chain reaction (RT-PCR) for the genotypic detection of rifampicin resistance in *Mycobacterium tuberculosis* isolates derived from diverse clinical specimens. The genotypic assay specifically targeted the rifampicin resistance-determining region (RRDR) of the *rpoB* gene, a well-established hotspot for mutations associated with rifampicin resistance.

Among the 37 MTB-positive cases analysed, a significant proportion exhibited mutations in key codons such as 516, 526, and 531, confirming the molecular basis of drug resistance. These findings reinforce the value of RRDR-targeted molecular testing as a rapid and accurate method for detecting rifampicin resistance. The detection of a mixed mutation pattern in one case also highlights the ability of genotypic methods to identify heteroresistance or mixed-strain infections, which may be missed by conventional techniques.

The use of the Truenat® microchip-based RT-PCR system allowed for DNA extraction, amplification, and mutation detection within a clinically relevant turnaround time. This rapidity makes genotypic diagnostics particularly advantageous in extra pulmonary TB cases, where bacterial load is often low, and timely diagnosis is critical. The internal controls and mutation-specific probe design ensured high specificity and minimized false interpretations.

Advances in Genotypic TB Diagnostics in India: Role of Truenat and Ni-kshay

India, being the country with the highest TB burden, has made significant investments in modernizing TB diagnostics. Recognizing the limitations of smear microscopy and culture, the National Tuberculosis Elimination Programme (NTEP) has prioritized molecular diagnostics to enhance early detection, especially of drug-resistant TB (DR-TB).

Truenat® is a microchip-based, battery-operated real-time PCR platform developed by Molbio Diagnostics in India. It has been endorsed by WHO as an alternative to GeneXpert, especially suitable for decentralized settings. It detects *M. tuberculosis* and rifampicin resistance (via *rpoB*) within 1–2 hours.

Ni-kshay (short for Nikshan–"elimination") is a digital tool managed by India's Ministry of Health & Family Welfare that, Tracks all notified TB cases across public and private sectors and Integrates lab results, treatment initiation, drug resistance status, and patient follow-up.

Together, Truenat and Ni-kshay represent India's dual strategy to improve early diagnosis and ensure continuity of care through digital data integration. Your study's RT-PCR work aligns well with this national vision.

APPENDIX

Drug resistance mechanism:

- Rifampicin is primarily caused by the by mutations in the **rpoB** gene, especially within the RRDR (codons 509–533).
- Early detection of MDR-TB is crucial for effective treatment and to prevent transmission.
- Molecular tests targeting RRDR mutations provide a good surrogate marker for rifampicin resistance and, by extension, MDR-TB.

Sample Processing and experimental workflow:

- Decontaminated using NALC-NaOH.
- DNA extracted using Trueprep® Auto Universal Cartridge.
- RT-PCR Amplification:
- Target genes: rpoB RRDR region (Codons 509–533)
- Platform Used: Truelab® Real-Time Quantitative micro PCR

Data Interpretation:

- Presence of mutations inferred from Ct shifts.
- Rifampicin resistance detected in 10 out of 37 positive cases.
- 4 representative isolates sequenced for rpoB mutations.

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This study aimed to evaluate the effectiveness of RT-PCR-based genotypic methods, particularly targeting the rpoB gene, in detecting rifampicin resistance among clinical isolates of Mycobacterium tuberculosis." The study seeks to evaluate the drug resistance patterns of clinical isolates against rst-line anti-tubercular drugs—speci cally isoniazid (INH) and rifampicin (RIF).
Molecular (genotypic) methods will be employed to identify the speci c genetic mutations conferring resistance, primarily within the rpoB gene (for rifampicin)
3.2 Speci c Objectives
1. To isolate Mycobacterium Tuberculosis (MTB) from clinical specimens collected from hospitalized patients suspected of pulmonary or extra pulmonary tuberculosis.
2. To detect gene mutations associated with drug resistance in phenotypically resistant isolates through genotypic methods

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ABSTRACT

Background:

Tuberculosis (TB) remains one of the world's leading infectious diseases, and the emergence of multidrug-resistant TB (MDR-TB), particularly resistance to rifampicin and isoniazid, has signi cantly complicated its management and control. Rifampicin resistance, which serves as a surrogate marker for MDR-TB, is primarily caused by mutations within the Rifampicin Resistance Determining Region (RRDR) of the rpoB gene. Rapid and accurate detection of rifampicin resistance is essential for timely initiation of second-line anti-TB treatment. This study aimed to assess the correlation between conventional phenotypic drug susceptibility testing (DST) and genotypic analysis targeting rpoB mutations in Mycobacterium tuberculosis (MTB) isolates.

Objectives:

To compare phenotypic and genotypic methods for the detection of rifampicin resistance in MTB isolates, evaluate the mutation patterns within the rpoB RRDR region, and analyze their diagnostic and clinical relevance.

Methods:

A total of 37 MTB-positive clinical specimens were included in the study. Genotypic analysis was performed on four representative isolates through ampli cation and molecular characterization of the rpoB gene, focusing on codons 509–533, which constitute the RRDR. Mutation patterns were examined.

Results:

Out of the 37 MTB-positive cases, 10 (27%) were identi ed as rifampicin-resistant , 13 cases (35.1%) were found to be sensitive.. Genotypic analysis of the four selected isolates showed resistance-associated mutations within the RRDR of the rpoB gene. Case 01 exhibited a mutation at codon 521, Case 02 at codon 516, Case 03 at codon 526, while Case 04 showed a mixed mutation pattern involving multiple codons. All detected mutations were consistent with known resistance-associated regions, con rming the RRDR as a critical hotspot for rifampicin resistance. The presence of a mixed mutation pattern in one case suggested possible heteroresistance or mixed-strain infection, which could complicate phenotypic interpretation and treatment response.

Conclusion:

This study highlights a high degree of correlation between genotypic and phenotypic pro les in detecting rifampicin resistance in MTB. Genotypic methods offer rapid, sensitive detection of common mutations and are especially valuable in time-critical settings. Phenotypic methods, despite being slower, remain essential for con rming resistance expression and detecting non-RRDR-mediated resistance.